METHODS FOR MAKING RECOMBINANT PROTEINS USING APOPTOSIS INHIBITORS

RELATED APPLICATIONS

This is a non-provisional application claiming priority under Section 119(e) to provisional application no. 60/156,232, filed Sep. 27, 1999, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The present invention relates generally to improved methods of making recombinant proteins using one or more apoptosis inhibitors.

BACKGROUND OF THE INVENTION

Control of cell numbers in mammals is believed to be determined, in part, by a balance between cell proliferation and cell death. One form of cell death, sometimes referred to as necrotic cell death, is typically characterized as a pathologic form of cell death resulting from some trauma or cellular injury. In contrast, there is another, "physiologic" form of cell death which usually proceeds in an orderly or controlled manner. This orderly or controlled form of cell death is often referred to as "apoptosis" [see, e.g., Barr et al., *Bio/Technology,* 12:487–493 (1994); Steller et al., *Science,* 267:1445–1449 (1995)]. Apoptotic cell death naturally occurs in many physiological processes, including embryonic development and clonal selection in the immune system [Itoh et al., *Cell,* 66:233–243 (1991)].

Control of cell numbers in cell culture and bioreactors is also a balance between cell proliferation and cell death. There have been reports in the literature indicating cell death in bioreactors can be an apoptotic process [Suzuki E., et al., *Cytotechnology,* 23:55–59 (1997); Al-Rubeai, M. and Singh R. P, *Curr. Opin. Biotech,* 9:152–156 (1998)]. It has been described that the apoptotic process may be induced by nutrient deprivation [Franek F. and Chládkova-Šrámková K., *Cytotechnology,* 18:113–117 (1995); Mercille S. and Massie B., *Biotechnol. Bioeng.,* 44:1140–1154 (1994); Singh R. P., et al., *Biotechnol. Bioeng.,* 44:720–726 (1994)], serum deprivation [Singh R. P., et al., *Biotechnol. Bioeng.,* 44:720–726 (1994); Zanghi A., et al., *Biotech. Bioeng.,* 64:108–119 (1999)] or other controllable parameters of cell culture in bioreactors, but is not controlled fully because of bioreactor mechanics, a lack of full understanding of necessary culture parameters, or other undetermined causes.

As presently understood, the apoptosis or cell death program contains at least three important elements—activators, inhibitors, and effectors; in *C. elegans,* these elements are encoded respectively by three genes, Ced-4 4, Ced-9 and Ced-3 [Steller, *Science,* 267:1445 (1995); Chinnaiyan et al., *Science,* 275:1122–1126 (1997); Wang et al., *Cell,* 90:1–20 (1997)]. Two of the TNFR family members, TNFR1 and Fas/Apo1 (CD95), can activate apoptotic cell death [Chinnaiyan and Dixit, *Current Biology,* 6:555–62 (1996); Fraser and Evan, *Cell;* 85:781–784 (1996)]. TNFR1 is also known to mediate activation of the transcription factor, NF-KB [Tartaglia et al., *Cell,* 74:845–853 (1993); Hsu et al., *Cell,* 84:299–308 (1996)]. In addition to some ECD homology, these two receptors share homology in their intracellular domain (ICD) in an oligomerization interface known as the death domain [Tartaglia et al., supra; Nagata, *Cell,* 88:355 (1997)]. Death domains are also found in several metazoan proteins that regulate apoptosis, namely, the Drosophila protein, Reaper, and the mammalian proteins

referred to as FADD/MORT1, TRADD, and RIP [Cleaveland and Ihle, *Cell,* 81:479–482 (1995)].

Upon ligand binding and receptor clustering, TNFR1 and CD95 are believed to recruit FADD into a death-inducing signaling complex. CD95 purportedly binds FADD directly, while TNFR1 binds FADD indirectly via TRADD [Chinnaiyan et al., *Cell,* 81:505–512 (1995); Boldin et al., *J. Biol. Chem.,* 270:387–391 (1995); Hsu et al., supra; Chinnaiyan et al., *J. Biol. Chem.,* 271:4961–4965 (1996)]. It has been reported that FADD serves as an adaptor protein which recruits the Ced-3-related protease, MACH-alpha/FLICE (caspase 8), into the death signaling complex [Boldin et al., *Cell,* 85:803–815 (1996); Muzio et al., *Cell,* 85:817–827 (1996)]. MACH-alpha/FLICE appears to be the trigger that sets off a cascade of apoptotic proteases, including the interleukin-1beta converting enzyme (ICE) and CPP32/Yama, which may execute some critical aspects of the cell death programme [Fraser and Evan, supra].

It was recently disclosed that programmed cell death involves the activity of members of a family of cysteine proteases related to the *C. elegans* cell death gene, ced-3, and to the mammalian IL-1-converting enzyme, ICE. The activity of the ICE and CPP32/Yama proteases can be inhibited by the product of the cowpox virus gene, crmA [Ray et al., *Cell,* 69:597–604 (1992); Tewari et al., *Cell,* 81:801–809 (1995)]. Recent studies show that CrmA can inhibit TNFR1- and CD95-induced cell death [Enari et al., *Nature,* 375:78–81 (1995); Tewari et al., *J. Biol. Chem.,* 270:3255–3260 (1995)].

As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor, NF-KB [Tewari et al., *Curr. Op. Genet. Develop.,* 6:39–44 (1996)]. NF-KB is the prototype of a family of dimeric transcription factors whose subunits contain conserved Rel regions [Verma et al., *Genes Develop.,* 9:2723–2735 (1996); Baldwin, *Ann. Rev. Immunol.,* 14:649–681 (1996)]. In its latent form, NF-KB is complexed with members of the IKB inhibitor family; upon inactivation of the IKB in response to certain stimuli, released NF-KB translocates to the nucleus where it binds to specific DNA sequences and activates gene transcription.

For recent reviews of such signaling pathways, see, e.g., Ashkenazi et al., *Science,* 281:1305–1308 (1998); Nagata, *Cell,* 88:355–365 (1997).

To date, there have been conflicting reports as to the effects of caspase inhibitors and expression of anti-apoptotic genes on cultured recombinant cells. For instance, Murray et al., *Biotech. Bioeng.,* 51:298–304 (1996) describe that overexpression of bcl-2 in NSO myeloma cells failed to affect the decline phase characteristics of the cultured cells. Other investigators have found, in contrast, that bcl-2 can be effective in preventing different cell lines from death under cell-culture conditions [see, e.g., Itoh et al., *Biotechnol. Bioeng.,* 48:118–122 (1995); Mastrangelo et al., *TIBTECH,* 16:88–95 (1998); Simpson et al., *Biotechnol. Bioeng.,* 54:1–16 (1997); Singh et al., *Biotechnol. Bioeng.,* 52:166–175 (1996)]. Goswami et al., *Biotechnol. Bioeng.,* 62:632–640 (1999) report that they found that the caspase inhibitor, z-VAD-fmk, was unable to substantially extend the life of a serum-free culture of CHO cells.

SUMMARY OF THE INVENTION

The present invention is based on Applicants' findings that employing one or more apoptosis inhibitor(s) in recom-